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Ketose Induced Respiratory Inhibition in Isolated Hepatocytes

P. Martínez, J. M. Carrascosa and I. Núñez de Castro*

Departamento de Bioquímica
Facultad de Ciencias
29071 Málaga (Spain)

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The addition of 10 mM fructose or 10 mM tagatose to a suspension of hepatocytes caused respiratory inhibition, whereas no change in oxygen uptake was observed following the addition of glucose. However, incubations in the presence of fructose showed a high, aerobic glycolytic activity. Tagatose is phosphorylated to tagatose 1-phosphate but is not further metabolized by cell free liver extract. Moreover, the addition of fructose to glucagon treated cells also caused the Crabtree-like effect. The concentration of adenine nucleotides and inorganic phosphate (Pi) in the mitochondrial and cytosolic compartments during incubation (time 30 min) was determined by the digitonin fractionation procedure. In the presence of 10 mM fructose or tagatose, the total adenine nucleotide pools decreased by 40%; however, glucose produced no change. The addition of ketoses diminished the asymmetric distribution of extramitochondrial $(ATP/ADP)_e$ ratio and intramitochondrial $(ATP/ADP)_i$ ratio. At the same time the total mitochondrial Pi fell from 17 mM to 6-7 mM. The mitochondrial membrane potential (−161 mV) in the presence of fructose showed no changes during the 30 min experimental period. An increase in the $NADH/NAD^+$ ratio was observed. These results suggest that in hepatocytes the inhibition of respiration is not necessarily linked with the enhanced aerobic glycolysis, by competition for common substrates.

Key words: Fructose, Hepatocytes, Respiration, Cellular compartmentation.

HASSINEN and YLIKAHARI (10) were the first to describe fructose induced inhibition of respiration in perfused rat liver. Then later, CHICO *et al.* (8) reported the metabolic circumstances in which this fructose induced, Crabtree-like effect takes place in isolated hepatocytes. In developing animals, the inhibition of respiration induced by fructose appears after weaning, as soon as the cells can catabolize fructose through the Hers pathway (7). In liver regenerating after partial hepatectomy, a decreased cellular respiratory capacity was observed, in which fructose did not modify the oxygen uptake (23).

The inhibition of respiration induced by fructose in hepatocytes isolated from fed rats is coincident with an increase of

* To whom correspondence should be addressed.

aerobic fructose break-down; nevertheless, the addition of tagatose to a suspension of hepatocytes also produced an inhibition of cellular respiration, very similar to that seen with fructose, but without the highly active aerobic glycolysis (22).

In this paper, the respiration rate, glycolysis and adenine nucleotide concentrations in the cytosol and mitochondria were determined during the incubation in different hexoses. The addition of ketoses to a suspension of hepatocytes caused a decrease in the total levels of adenine nucleotides (21, 31), and was accompanied by the diminution of the asymmetry of the ATP/ADP ratio between the cytosolic and mitochondrial compartments.

Materials and Methods

Chemicals. — D-glucose, D-fructose and digitonin were from E. Merck, Darmstadt (Germany). D-tagatose was from Sigma. Bovine serum albumin was purchased from Metrix-Armour Pharmaceutical Company (U.S.A). Enzymes and coenzymes were supplied by Boehringer Mannheim and Sigma. Other chemicals were reagent-grade products. Radioactive preparations, Br[^{14}C]-TPP (specific radioactivity 31.4 mCi/mmol), [^{14}C]-inulin (specific radioactivity 15.4 mCi/mmol) and K[^{14}C]-SCN (specific radioactivity 56.3 mCi/mmol), were from The Radiochemical Centre (Amersham). Silicone oils AR 200 and Ar 20 were bought from Wacker Chemie, München.

Preparation and incubation of isolated hepatocytes. — Female Wistar rats of 250-300 g weight, fed *ad libitum*, were housed at 22°C and accomodated to an 08.00-22.00 hour light-dark schedule.

Hepatocyte isolation and incubation followed the procedure reported previously (6). The cellular integrity was always carefully checked. At 90 min after isolation, at least 87% of the cells were intact as shown by the erythrosine exclusion test; the leakage of lactate dehydrogenase was about 20% of total activity.

Fractionation procedure. — The cell fractionation method was as follows: a 3 ml polypropylene centrifuge tube was loaded with 0.5 ml of 1 M $HClO_4$ and 1 ml of silicone oil mixture, types AR 200 and AR 20 (2:1, w/w). The upper layer consisted of 0.5 ml of a medium containing 0.25 M mannitol, 3 mM EDTA, 20 mM MOPS (4-morpholinepropanesulphonic acid) at pH 7.0; plus 2 mM digitonin when necessary. The tube was kept at 0°C. A portion (0.5 ml) of the cell suspension (equivalent to 10 mg dry weight) was rapidly mixed with the upper layer by a Gilson Pipetman P 1000 with a disposable ben tip. Ten seconds after sampling, the tube was centrifuged for 30 s at 4,000 × *g* in a MSE centrifuge, model Super-minor. The values obtained for digitonin treated cells represent the mitochondrial compartment. The values obtained for whole cells, minus those found in the digitonin treated cell pellet, were assumed to represent the cytosolic compartment. For the marker enzymes, determination 0.5 ml of 1% Triton X-100, 0.33 M sucrose solution was substituted for the $HClO_4$ solution. The digitonin was purified by the method described by JANSKI and CORNELL (14).

The validity of the method was tested using lactate dehydrogenase as a cytosolic marker and citrate synthase and glutamate dehydrogenase as mitochondrial markers. The exposure time (10 s) and the concentration of digitionin (1 mM) were shortened in comparison with the method described by AKERBOOM *et al.* (1).

Assay of the flow through aldolase. — The metabolic flow through aldolase was determined by measuring the triose phosphates formed by a partially puri-

fied, cell free extract incubated in the presence of different concentrations of fructose or tagatose. The incubation medium was 4.2 mM ATP, 4.2 mM magnesium acetate, 1 M potassium acetate and 42 mM NaF. The crude extract was partially purified by centrifugation at 30,000 × *g* for 20 min and the gel filtered through a Sephadex G-25 column.

Enzyme activities and metabolite content determinations. — Metabolites were determined by standard enzymatic procedures (2). Glutamate dehydrogenase was assayed according to SWAISGOOD *et al.* (29), lactate dehydrogenase according to BERGMEYER *et al.* (3) and citrate synthase, as described elsewhere (25). Inorganic phosphate was determined as described by LANSETTA *et al.* (20).

Oxygen uptake was measured using the procedures described by CHICO *et al.* (8).

Mitochondrial membrane potential determination. — Mitochondrial membrane potential was calculated by determining the distribution of lipophilic labeled cation [^{14} C]-TPP^{+} in the incubation medium, and the cytosolic and mitochondrial water spaces, following the method described by HOEK *et al.* (13).

Results and Discussion

The addition of 10 mM fructose to a suspension of hepatocytes resulted in a 40% inhibition of respiration (fig. 1). No difference in oxygen uptake was observed when 10 mM glucose was added. The addition of fructose to glucose metabolizing cells caused the Crabtree-like effect; on the other hand, the addition of glucose to fructose metabolizing cells did not modify the slope of polarographic line (fig. 1, D and E). The profile of the line after the addition of 10 mM tagatose is very similar to that obtained by addition of fructose. A transient stimulation of respiration (about 1 min) was always detected, when fructose or tagatose were added to a suspension of hepatocytes metabolizing endogenous substrates. This effect was first observed by CHANCE and HESS (4) in ascites tumor cells. Thereafter, a new oxygen uptake steady state was reached which remained unchanged at least for 30 min. In cells aerobically incubated in the presence of fructose, the glycolytic flux is about 3 fold higher than in cells incubated in glucose (6). In the cells treated with glucagon a significant decrease in the production of lactate was observed (table I). Nevertheless, in these conditions, the presence of fructose caused an oxygen uptake decrease by 40%.

Figure 2 shows the production of triose phosphates by a partially purified liver extract incubated in different concentrations of tagatose and fructose. D-tagatose is a very good ketohexokinase substrate (12) and is converted into tagatose 1-phosphate which is not further metabolized in the liver. The low production of triose phosphates in the presence of tagatose indicates an interruption of the glycolytic flux at the level of liver aldolases.

The Crabtree effect and the aerobic glycolysis have been considered as two linked phenomena caused by competition for common substrates ADP or Pi, between the substrate-level phosphorylation and mitochondrial phosphorylation. The inverse relationship between respiration rate and glycolysis in many tissues supports the popular hypothesis of competition. The work of KOOBS (19) in tumor mitochondria emphazised the role of inorganic phosphates. On the other hand, GOSÁLVEZ *et al.* (9) using an artificial model, consisting of pyruvate kinase and coupled rat liver mitochondria, confirmed the early hypothesis of CHANCE and HESS (5) that there is competition for ADP. The above results indicate that the inhibition of respiration and the aer-

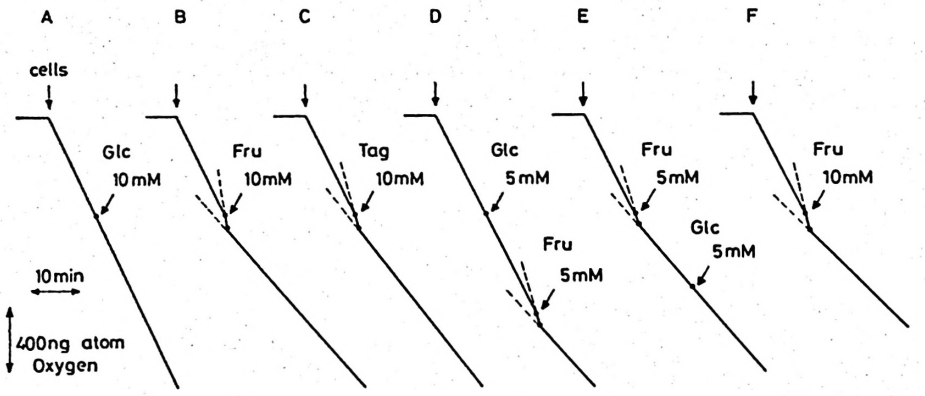

Fig. 1. *Effect of fructose and tagatose on oxygen uptake in hepatocytes isolated from well-fed rats.* The arrows on polarographic lines indicate the moments the additives were introduced into the electrode vessel; 10 mg dry weight of cells were used in each experiment; (F) cells preincubated with 2 μM glucagon for 30 min, this glucagon concentration was also maintained in the electrode vessel during the experiments. Glc: D-glucose, Fr: D-fructose, Tag: D-tagatose.

obic glycolysis are not necessarily linked.

The use of a cellular compartmentation technique to study the metabolic changes that occur following the addition of fructose or tagatose, and to compare them with those following the addition of glucose, may provide a new insight into the cytosolic and mitochondrial interplay in the cellular energy metabolism (27).

The addition of glucose to a suspension of hepatocytes did not change the total adenine nucleotide pool; on the contrary, the presence of ketoses induced a drop in the levels of adenine nucleotides which reached its minimum, 60% of the initial value, 5 min after incubation (30, 31). Figures 3, 4 and 5 show the distribution of ATP, ADP and Pi in the mitochondrial and cytosolic water spaces that occurred during incubation of hepatocytes isolated from well fed animals and incubated in 10 mM glucose, 10 mM fructose, or 10 mM tagatose. In the presence of glucose there were no significant changes

Table I. *Lactate production of isolated hepatocytes incubated in different media.* Figures are means of 4-8 different experiments, they represent the lactate production in nmol/mg dry weight^{-1} ± S. E. Statistical significance against the same incubation time in the presence of 10 mM of fructose was measured by Student's *t* test.

Incubation time (min)	Fructose (10 mM)	Glucose (10 mM)	Tagatose (10 mM)	5 mM Glc + 5mM Fru	10 mM Fru+ 2 μM Glucagon
0	47 ± 6	47 ± 6	47 ± 6	47 ± 6	47 ± 6
0.5	86 ± 15	38 ± 8	32 ± 4	nd	nd
1	87 ± 16	42 ± 10	36 ± 4	nd	nd
5	83 ± 13	47 ± 8	52 ± 9	nd	nd
15	120 ± 15	52 ± 10	47 ± 7	nd	80 ± 12[a]
30	180 ± 11	64 ± 7	58 ± 5	nd	nd
60	336 ± 28	126 ± 25	120 ± 11	260 ± 30	242 ± 28[b]

(a) $p < 0.05$; (b) $p < 0.025$; nd: not determined.

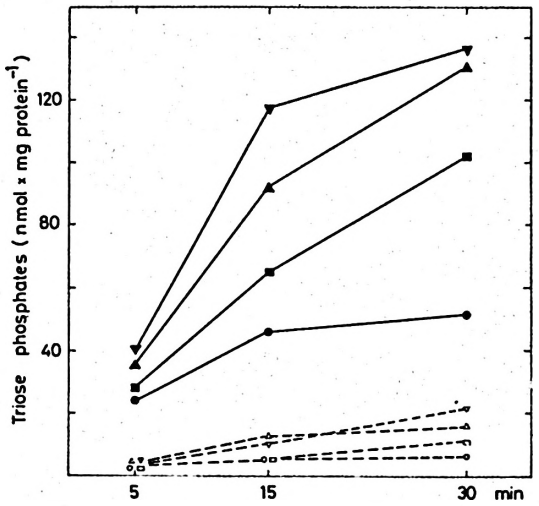

Fig. 2. *Production of triose phosphates from fructose and tagatose by partially purified cell-free liver extract.*
The incubation medium was (●) 0.5 mM, (■) 1 mM, (▲) 5 mM and (▼) 10 mM fructose; (○) 0.5 mM, (□) 1 mM, (△) 5 mM and (▽) 10 mM tagatose.

in the cytosolic and mitochondrial concentrations of ATP and ADP. An asymmetric distribution of the ATP/ADP ratio between the mitochondrial and cytosolic compartments was observed, in agreement with the results of other authors (11, 17, 27, 28).

A completely reverse situation arose when the cells were incubated in the presence of fructose. Thirty seconds after the addition of ketose, the concentration of Pi had drastically decreased in both compartments. In the cytosol, a significant decrease in ATP and a significant increase in ADP were found. These changes were simultaneous with the initial burst in the respiration rate. Similar results were observed in the presence of 10 mM tagatose.

After 5 min of incubation in the presence of fructose, the phosphorylation potential difference between cytosolic and mitochondrial compartments, or translocation energy (15), decreases from 11.9 kJ × mol^{-1} to 3.7 kJ × mol^{-1}. Thus, the

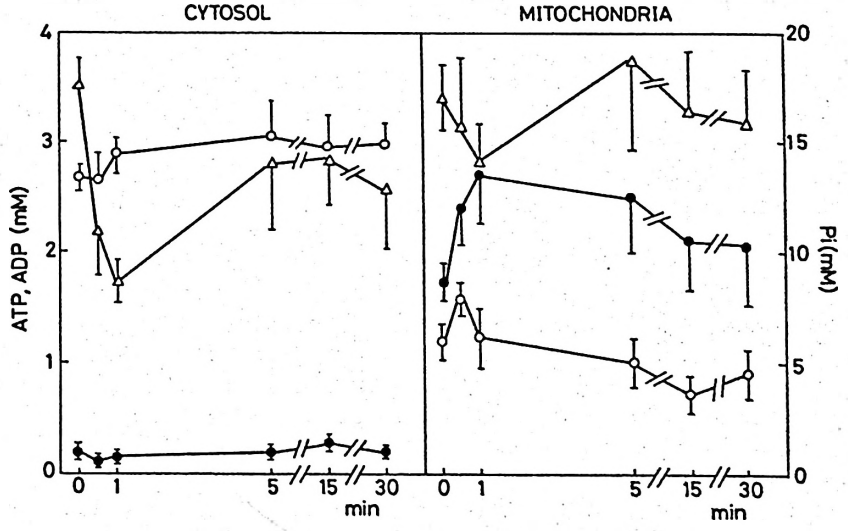

Fig. 3. *ATP (○), ADP (●) and Pi (△) distribution in the cytosolic and mitochondrial compartments of isolated hepatocytes during incubation in 10 mM glucose.*
Values are means of six different experiments ± S. E. Cellular incubations (about 24 mg dry weight per 1.2 ml) were carried out at 37°C in 25 ml flasks under an atmosphere of 95% O_2 and 5% CO_2. The concentration of digitonin was 1 mM and the exposure time 10 s. The cytosolic and mitochondrial water spaces were assumed to be 2.0 and 0.21 ml/g dry weight (1).

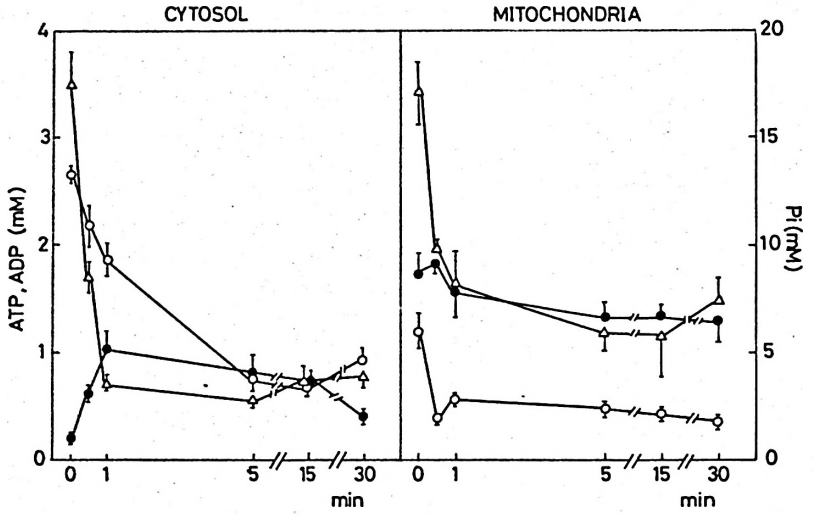

Fig. 4. *ATP (O), ADP (●) and Pi (△) distribution in the cytosolic and mitochondrial compartments of isolated hepatocytes during incubation in 10 mM fructose.*
Values are means of six different experiments ± S. E. Incubation conditions as in figure 3.

difference between the ATP/ ADP ratios in the extramitochondrial space and mitochondrial matrix was diminished (table II).

According to KLINGENBERG and ROTTENBERG (18) the ratio $(ATP/ADP)_e$: $(ATP/ADP)_i$ in isolated liver mitochondria correlates to the mitochondrial membrane potential. In the fructose incubated cells the membrane potential, determined by the distribution of lipophilic cation TPP^+, did not change during the 30 min of the experiment (table II). An average value of 161 mV was obtained.

In the presence of fructose, the mitochondria appear to be coupled, as can be deduced from the crossover analysis of the mitochondrial $NADH/NAD^+$ ratio during incubation according to the equilibrium reaction: $NADH/NAD^+ = K'_{eq}$ ([3-hydroxybutyrate]/[acetoacetate]) ($K'_{eq} = 4.93 \times 10^{-9}$ at 37°C, pH 7.0) (24). At 30 s a fall in NADH concentrations was observed simultaneously with the increase in the oxygen uptake (figure 1). After 60 s, when the inhibition of respiratory takes place, an increase in the $NADH/NAD^+$ ratio was observed.

These results suggest that, in the presence of ketose, the adenine nucleotide translocator system is substantially altered, in such a way that the electrical energy-dependent transport system is switched to the alternate energy independent process (16).

A significant decrease in the mitochondrial ADP concentrations in the presence of fructose or tagatose coincident with the decrease in the respiration rate, is found 5 min after the addition of ketoses. These results seem to confirm the hypothesis of CHANCE and HESS (5), that the inhibition of respiratory following the addition of glucose to to Ehrlich ascites cells, is due to a decrease of the mitochondrial phosphate acceptor, since the inhibition of respiratory in the whole cell, may be similar, in some way, to the change from mitochondrial state 3 to state 4.

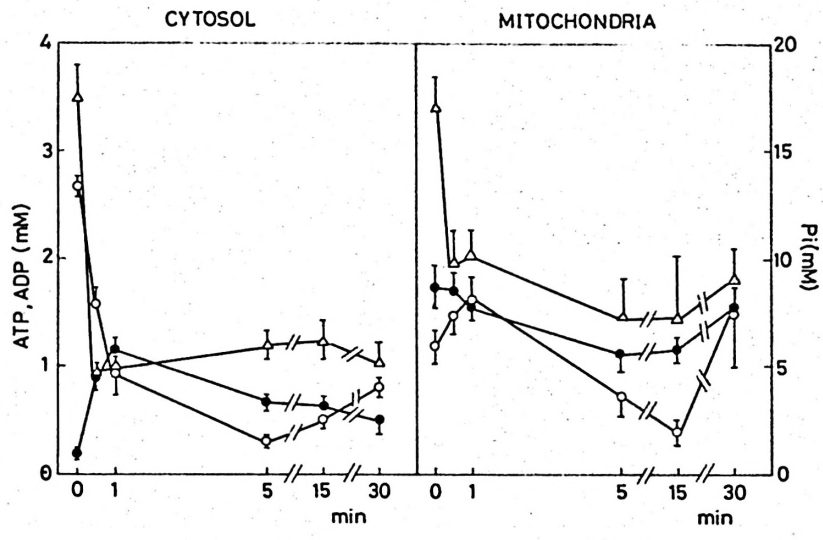

Fig. 5. *ATP (O), ADP (●) and Pi (△) distribution in the cytosolic and mitochondrial compartments of isolated hepatocytes during incubation in 10 mM tagatose.*
Values are means of six different experiments ± S. E. Incubation conditions as in figure 3.

However, in the presence of ketoses, the inorganic phosphates fell sharply in both compartments, although the concentration of total Pi in the mitochondria was always higher than the apparent K_m value of 1 mM reported for Pi in the oxidative phosphorylation process (26). This raises the question of the importance of Pi concentration in the control of mitochondrial respiration, a problem which has not yet been satisfactorily resolved. Our results give further evidence that the isolated hepatocytes are a good model for the study of the interactions taking place between cytosolic and mitochondrial compartments in the whole cell.

Table II. *Changes in asymmetrical distribution of ATP/ADP ratio between cytosolic and mitochondrial spaces, mitochondrial membrane potential (ΔΨ) and mitochondrial redox state of isolated hepatocytes incubated in different hexoses.*

Incubation time (min)	$(ATP/ADP)_e : (ATP/ADP)_i$			ΔΨ (−mV)[a]	NADH/NAD$^+$
	Glucose 10 mM	Fructose 10 mM	Tagatose 10 mM	Fructose 10 mM	Fructose 10 mM (xK'^{-1}_{eq})
0	20	20	20	168 ± 10	0.42
0.5	34	1.9	17.5	161 ± 5	0.35
1	44	0.8	4.5	153 ± 10	0.43
5	42	0.7	2.3	167 ± 16	0.64
15	36	1.8	3	153 ± 15	0.80
30	37	1.7	7.3	164 ± 15	0.93

(a) Determined by distribution of lipophilic cation [$^{-14}$C]TPP$^+$, as described in methods.

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Resumen

La incubación de hepatocitos en presencia de fructosa 10 mM, o de tagatosa 10 mM, induce una inhibición de la respiración celular, no observándose ningún cambio en el consumo de oxígeno al añadir glucosa. Sin embargo, las células incubadas en presencia de fructosa 10 mM muestran una elevada actividad glucolítica. La tagatosa, que es fosforilada a tagatosa 1-fosfato, es muy débilmente metabolizada por un extracto hepático parcialmente purificado. La adición de fructuosa a células tratadas con glucagón también induce un efecto similar al efecto Crabtree. Se ha determinado, por el procedimiento de fraccionamiento con digitonina, la concentración de nucleótidos de adenina y fosfato inorgánico (Pi) en los compartimentos citosólico y mitocondrial durante el tiempo de incubación (30 min). En presencia de fructuosa 10 mM o de tagatosa 10 mM, la cantidad total de nucleótidos de adenina disminuye un 40%; mientras que la glucosa no induce cambios en la concentración total de nucleótidos. La adición de cetosas disminuye la distribución asimétrica existente entre la razón $(ATP/ADP)_e$ extramitocondrial y la razón $(ATP/ADP)_i$ intramitocondrial. Simultáneamente, la cantidad de Pi mitocondrial baja de 17 mM a 6-7 mM; el potencial de la membrana mitocondrial (−161 mV) no muestra variación, en presencia de fructosa, durante los 30 min de duración del experimento. Igualmente se observó un incremento de la razón $NADH/NAD^+$. Estos resultados sugieren que en hepatocitos aislados de rata alimentada, la inhibición de la respiración y la glucolisis aerobia son dos procesos no necesariamente ligados al competir por sustratos comunes.

Palabras clave: Fructosa, Hepatocitos, Respiración, Compartimentación celular.

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